行政院國家科學委員會專題研究計畫 成果報告

以心包膜發炎反應誘發心肌血管新生之研究
研究成果報告(精簡版)

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報告內容

（計畫名稱）

Study on the induction of myocardial angiogenesis by pericardial inflammatory reaction – A porcine model

以心包膜發炎反應誘發心肌血管新生之研究 – 豬之研究模式

Introduction

Despite the advances in revascularization therapy for coronary artery disease with either surgical bypass surgery or percutaneous transcatheter interventional technique, a certain number of patients are suffering from severe ischemia but are poor candidates for these therapeutic modalities (1-3). For these patients, the disability resulting from severe limiting angina is substantial. One potential strategy of therapy for these patients is the creation of new channels for blood supply to the ischemic region, transmyocardial revascularization (TMR) as an example (1-3). Yet, early series of TMR reported a high (5-9%) perioperative mortality with even higher overall morbidity (3-7). An alternative option is the induction of growth of new blood vessels to the ischemic myocardium, or angiogenesis (1,3,7-11).

Although a number of potential angiogenic agents have been identified to enhance the natural process of new vessel development, only two growth factors, fibroblastic growth factor (FGF) (12) and vascular endothelial growth factor (VEGF) (13,14) have been applied in clinical trials on patients with refractory ischemia. These agents have been administered to the patients through different routes, including intravenous, intracoronary, intramyocardial, and intrapericardial routes (12,13).

Although angiogenesis therapy using either systemic or local delivery of growth factors or surgical laser revascularization remains active areas of investigation, the results have been found somewhat disappointing to date (15). In one prospective, multicenter, randomized trial in patients with class III or IV angina caused by nonrecanalizable chronic total occlusion of coronary arteries, percutaneous TMR did not bring about a greater reduction in angina, more improvement in exercise duration or survival free of adverse cardiac events, as compared with maximal medical treatment only (16).

Given the typically long course of new vessel development, most attempts to stimulate myocardial angiogenesis have used methods of prolonged growth factor delivery, including gene therapy, continuous infusions, repeated injections, or sustained release polymers (17). The pericardial space may potentially serve as a drug

delivery reservoir that may be used to deliver therapeutic agents to the heart with persistent retention of the delivered drugs in the pericardial space for a prolonged action (17,18).

In a previous study, we injected minocyclin to pericardial sac of pigs, in which myocardial infarction (MI) had been produced, to induce pericardial adhesion. The hypothesis for that experiment was that, in animals suffering from MI, constraint of the heart by inducing pericardial adhesion to the heart would prevent deteriorating ventricular dilatation ("remodeling"). In that experiment, we observed that in pigs with pericardial injection of minocyclin, there was active formation of new microvasculature in the myocardium. We propose that, in response to pericardial application of some stimulants, like minocyclin, the resulted response with active angiogenesis in the diseased heart may be beneficial in providing more blood supply to the myocardium, which may involve both the normal and the ischemic myocardium.

The mechanisms for the induction of active angiogenesis in this animal model are not clear. We proposed that minocyclin-induced inflammatory reaction in the pericardium and myocardium may play a major role. Inflammation is closely intertwined with the process of repair and has been demonstrated to be associated with angiogenesis (19-21). The cellular and molecular changes involved in inflammation have been extensively reviewed by Kumar, Abbas and Fausto (22).

In this research, we investigated the effects of pericardium-applied minicyclin on the induction of myocardial angiogenesis. We tested different doses of minocyclin to produce various degrees of pericardial adhesion and induction of angiogenesis.

Methods and Materials

A. Animals for study

Mini-pigs were used in this study. Acute MI was induced in all pigs. One week after induction of MI, the pigs were randomly assigned to one of the following study groups: 1) Pericardial adhesion/inflammation group, in which minocyclin at a dose of 2, 4 or 8 mg/kg body weight was injected into pericardial sac; 2) Control group, in which pericardial injection with normal saline was done.

B. Induction of acute MI

Mini-pigs with body weights between 9.8-28 kilograms were used in this experiment. Mini-pigs were anesthetized with intramuscular injection of ketamine

(4-5 ml, 50 mg/ml) and atropine (0.5-1.0 mg). An intravenous route was set-up on ear vein for injection of drugs, including supplemental anesthetics, such as propofol and succinylcholine, and drugs for treating arrhythmia at resuscitation. The pigs were under control respiration with a Harvard respirator with room air by endotracheal intubation.

The baseline electrocardiogram (ECG) was then taken. By skin cut-down, right femoral artery was catheterized with a 6F sheath. After blood pressure recording, through the sheath, a 6 Fr right Judkins catheter was advanced to engage the left coronary artery. After confirmation of the coronary anatomy by contrast medium injection, the catheter was further advanced into the left anterior descending coronary artery (LAD) to about the mid way between the coronary orifice and the apex. There, through the catheter, a Gianturco coil (3 mm in diameter and 3 cm in length, Cook Group Inc.) was deployed to occlude the coronary artery (23,24). The pigs were then monitored for hemodynamics and ECG changes.

Life-threatening arrhythmias (VT/VF), when occurs, were treated with cardio-pulmonary resuscitation, drug administration (lidocaine and adrenaline), and DC cardioversion. When the pigs' condition become stable, parenteral antibiotics (IV cefamezin and IM gentamycin) were injected. Pigs were then sent back to animal room for further care. A second ECG was taken to document the ECG changes of AMI before leaving the catheterization laboratory. The animals were treated with Keflex for 1 week and acetaminophen to relief the wound pain.

C. Pericardial access and drug injection

One week after induction of AMI, the pigs were again anesthetized and put on artificial respiration. Mediastinum was entered by a minimal subxiphoid skin cutdown (about 5 cm in length). Under direct vision, a No. 20 intravenous catheter was inserted into the pericardial sac for administration of drugs. After drug injection, the intravenous catheter was removed and any leakage of drugs from pericardial sac was inspected. The skin wound was then closed and the pigs returned to the animal room for further care. Antibiotics and analgesic were given as above. As mentioned, the pigs were randomly assigned to receive either normal saline or different doses of minocyclin (8 mg 4 mg or 2 mg per kilogram body weight).

D. Pathological observation

Four weeks after induction of AMI (3 weeks after intrapericardial drug injection), the pigs were sacrificed. The changes of the mediastinum, the pericardium, and the

heart were examined, including the infarct size, pericardial change and adhesion, the thickness of the infarction area, and the drained weight of the heart. The myocardial samples were then taken from around the infarct area and the normal, non-infarct area for pathological examination. Parts of the specimens were preserved in formalin and the remaining parts in liquid nitrogen.

Tissue samples were immersed in 10% formalin fixative overnight. Following dehydration through a graded series of ethanol, the samples were embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin stain. Histological sections were observed and photographed under the light microscope for counting of vasculature.

E. Evaluation of angiogenesis

The significant increased number of CD31-positive blood vessels in the stroma indicated increased angiogenesis in response to inflammatory stimuli (25). One paraffin-embedded section, at a thickness of 5 μm, was obtained from each group. Rat monoclonal antibody against porcine CD31 (Research Diagnostics, Inc., Flanders, NJ) was used for the detection of microvessels. Immunohistochemical staining was performed by a streptavidin-biotin peroxidase method. Endogenous peroxidase was inactivated by immersion of sections in 3% H2O2 in isotonic PBS for 10 min. After immersion of the sections in a blocking agent solution for 20 min (Protein Block Serum-Free, Dako Japan), sections were incubated with the anti-CD31 antibody for 2 hr. After being washed in PBS, sections were incubated with biotinylated-rabbit antirat IgG (Zymed Laboratories, Inc., South San Francisco, CA) for 20 min at room temperature, washed in PBS, and incubated with peroxidase-labeled streptavidin for 20 min at room temperature. The sections were finally treated with 0.03% 3,3’-diaminobenzidine (Dojin, Kumamoto, Japan) containing 0.005% hydrogen peroxidase and sodium azide. After immunohistochemical staining, angiogenesis was evaluated by counting the number of microvessels in the granulation tissue in five fields (x100), and the average number of microvessels was considered microvessel density.

F. The extent of inflammation examined by immunohistochemistry and Western blot

The roles of the inflammatory cytokines, especially interleukin (IL)-1β, are emphasized in inflammation-associated angiogenesis (26,27). The proposal was designed to examine and compare the expression of the IL-β protein among these groups by immunohistochemistry and Western blot. For immunohistochemistry,

paraffin-embedded section was used. The tissue section was deparaffinzed, rehydrated, washed with PBS, and treated with 5mg/ml serum albumin in PBS for 1 h to block non-specific binding. The section was incubated with goat anti-human IL-β primary antibody (1:15 dilutions, R&D systems, USA). Antibodies were localized by an indirect immunoperoxidase technique (avidin-biotin horseradish peroxidase complex) employing diaminobenzidine as a chromogen (Vector Lab, USA). Negative control was performed by omitting the incubation of the primary antibodies in the tissue sections. For Western blotting, the tissues from these groups were lysed for 1 h at 48°C with lysis buffer (0.5 M NaCl, 50 mM Tris, 1 mM EDTA, 0.05 % SDS, 0.5 % Triton X-100, and 1 mM PMSF) and centrifuged at 4,000g for 30 min at 48C. The supernatants were applied to 8% SDS±PAGE and transferred at room temperature by blotting to polyvinylidene difluoride (PVDF) membrane (NEN), which were then treated for 1 h at room temperature with PBS-Tween 20 (0.05%)/ 2% skim milk and separately incubated for 1 h at room temperature with goat anti-human IL-β primary antibody. After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-goat monoclonal antibodies. Antigen detection was performed via Chemiluminescence Reagent Plus (NEN) and exposure to Biomax MR film (Kodak).

RESULTS

Animal experiments

A total of 32 mini-pigs were put into experiments, among them 24 successfully accomplished the experiments, a 75% success rate. Of these experimental animals, 5 were injected with normal saline (control group) while the remaining pigs received intrapericardial minocyclin injection with a dose of 2 mg/kg, 4 mg/kg or 8 mg/kg, on 7 pigs, 7 pigs and 5 pigs, respectively (Table 1). The body weights for these animals were ranged between 9.6 Kg and 28 Kg, with a mean of 16.1$\pm$4.4 Kg. All pigs showed ECG changes compatible with acute anterior wall myocardial infarction (Fig. 1).

Table 2 shows the data about body weight at baseline and heart weight, pericardial adhesion and infarct size at sacrifice. All pigs except one with pericardial injection of minocyclin showed pericardial adhesion while all the pigs injected with normal saline revealed no pericardial adhesion. Table 3 shows the mean body weight and heart weight by experiment groups. The mean body weight was somewhat lower for the normal saline-injection group (all $p<0.005$) while the heart weight was

significantly lower for the normal saline group as comparing to the minocyclin-injection groups (all $p<0.005$), but the corrected heart weight (heart weight/body weight) was significantly different only for the 4 mg/kg group.

Gross observation of the heart and pericardium

All heart showed infarction changes of various sizes, located distal to the occlusion site of left anterior descending coronary artery (Fig. 2). The infarcts usually extended down to the apex and, in some pigs, even more posteriorly to some extent. In most pigs, the infarct size was small or moderate. In all 5 pigs received intrapericardial injection with normal saline, no pericardial adhesion was observed (Fig. 2-A). In contrast, pericardial adhesion was observed in all pigs receiving intrapericardial minocyclin injection (Fig. 2-B), except for one pig in which moderate amount of pericardial effusion with minimal pericardial adhesion was noted. About the extent of pericardial adhesion for the pigs receiving minocyclin intrapericardial injection, 2 pigs showed local adhesion while the remaining 17 pigs demonstrated diffuse pericardial adhesion.

The infarct size was arbitrarily assigned as small, moderate or large. We noted that the infarct size was small in 13 pigs, moderate in 10 pigs and large in only one pig. On cutting open the left ventricle longitudinally through the middle of the infarct area, the infracted myocardium was thinned in all pigs (Fig. 2-C) except for one pig in which the infarct area became much thickened with yellowish, fat-like infiltration (Fig. 3). The heart weights of the pigs ranged from 65 to 150 g (mean 110.7 ± 22.6 g) and the corrected heart weight (heart weight divided by body weight) ranged from 4.32 to 10.07, with an average of 7.17 ± 1.45 g/kg. Table 3 denotes the mean body weight and heart weight by experiment groups. Although pigs injected with minocyclin showed higher heart weight in comparing with pigs injected with normal saline (all $p<0.005$), the corrected heart weight was significantly different only between pigs with normal saline and pigs received 4 mg/kg minocyclin injection ($p<0.05$).

Microscopic observation of the heart

Microscopically, myocardial infarction was confirmed in all pigs. In order to detect the presence and to count the number of blood vessels in the infarct areas and the peri-infarct areas, we intended to use special staining in our experimental design. Yet, we found that specific anti-porcine antibodies against CD-31, CD-34 or von Willebrand factor were not commercially available. We tried many times to stain the myocardium using some antibodies not specific against porcine antigens and was fail to get staining of the myocardial specimens for evaluation.

Finally, we did analysis on myocardial specimens with H-E staining. We hope that we could soon find some specific antibodies so that we can complete our study in the future. The myocardial specimens are now still kept in stock, including liquid nitrogen storage.

In analyzing the specimens of H-E staining specimens, we noted significantly increased blood vessels in the infarct areas, especially for pigs receiving pericardial injection of minocyclin. Fig. 4 shows microscopic findings of some example pigs. Fig. 4-A is from a pig injected with normal saline. Some arterioles and microvessels could be identified in the infarct area. Fig. 4-B is a specimen from a pig with 2 mg/kg minocyclin injection. There are many microvessels appeared in this specimen. Fig. 4-C is obtained from a pig with 4 mg/kg minocyclin injection. There are many microvessles developed in this specimen. There are also venules developed in the infarct area. In observing the number of all vessels in the infarct area, we analyzed 2 pigs for each group. The averaged vessel numbers for one 200X microscopic field for the different groups are: N/S group: 44$\pm$16; minocyclin 2 mg/kg group, 55$\pm$18; minocyclin 4 mg/kg group, 62$\pm$20; minocyclin 8 mg/kg group, 55$\pm$16. It seemed that with pericardial injection of minocyclin would induce neovascular development in the infarct area, as comparing with normal saline injection.

DISCUSSION AND COMMENT

Our study was based on our incidental observation that in the animal model of infarct-induction experiment, in this case a pig model, pericardial inflammation might stimulate neovascular proliferation. This observation is interesting and may be clinically relevant. If we can induce more vessel growth in and around the infarct zone, we may reduce myocardial ischemia after myocardial infarction, which may result in improved left ventricular function with better prognosis.

In this experiment, we are successful in inducing myocardial infarction with subsequent pericardial injection of minocyclin or normal saline in 24 pigs. We intended to study the minocyclin-induced pericardial and myocardial inflammation for the induction of neovasculature development in or around the infarct area. Yet, we encountered a difficulty in immunohistochemically staining the pathological specimens for quantification of neovasculature. We used H-E-stained specimens for vessel counting instead, which are difficult for us to accurately count the vessel number. We will further try to find some specific antibody to complete the works. We expect that after successful staining of these specimens for vessel quantification we may obtain exciting results for publication.

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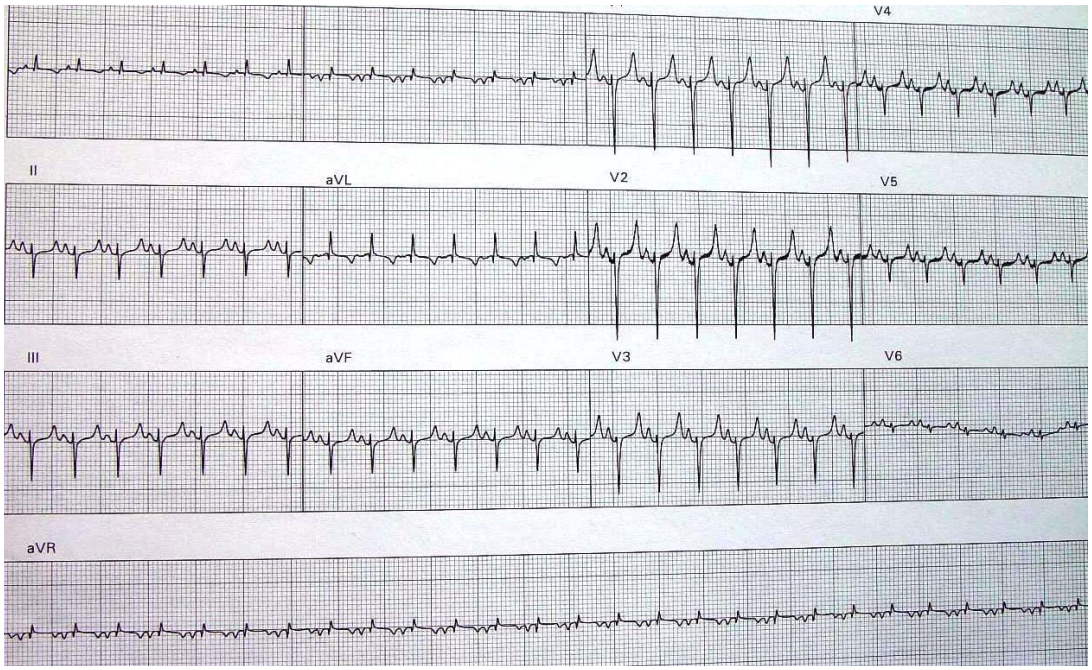
Figures (1-4) and Tables (1-3)

A



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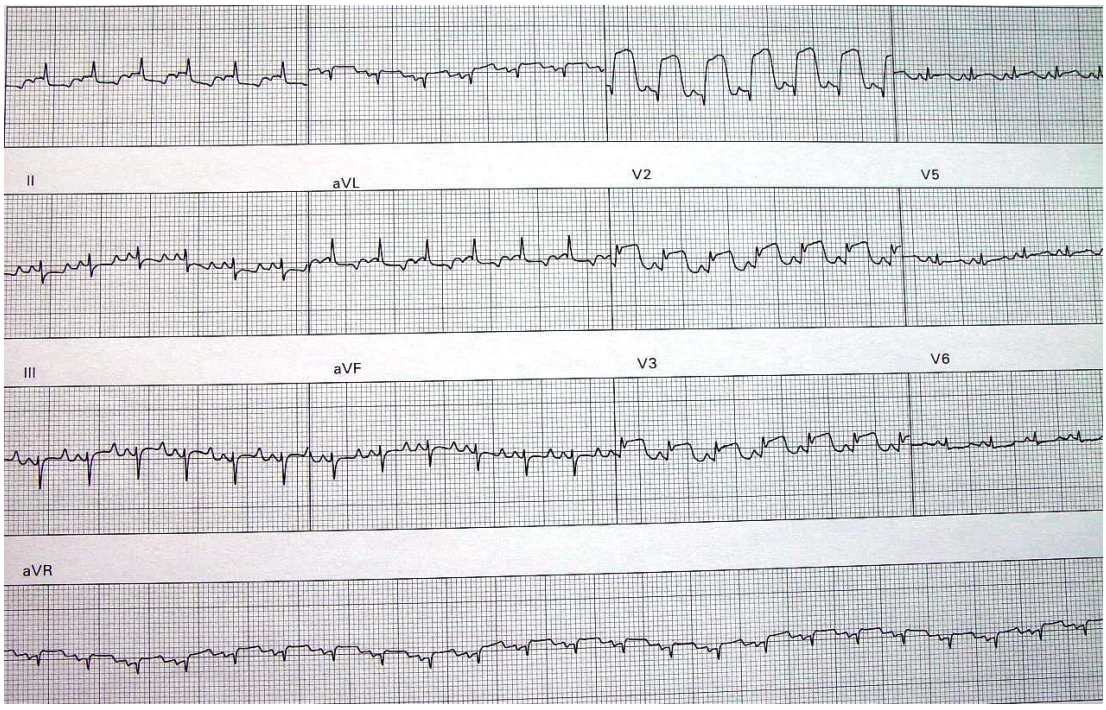
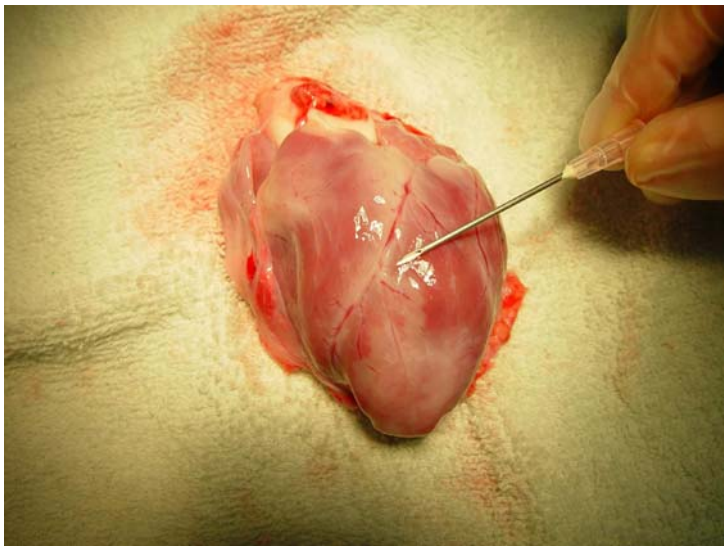


Fig 1. ECG at baseline (A) and after induction of acute myocardial infarction (B) showing typical anterior myocardial infarction: marked ST elevation in leads V1-3.

A



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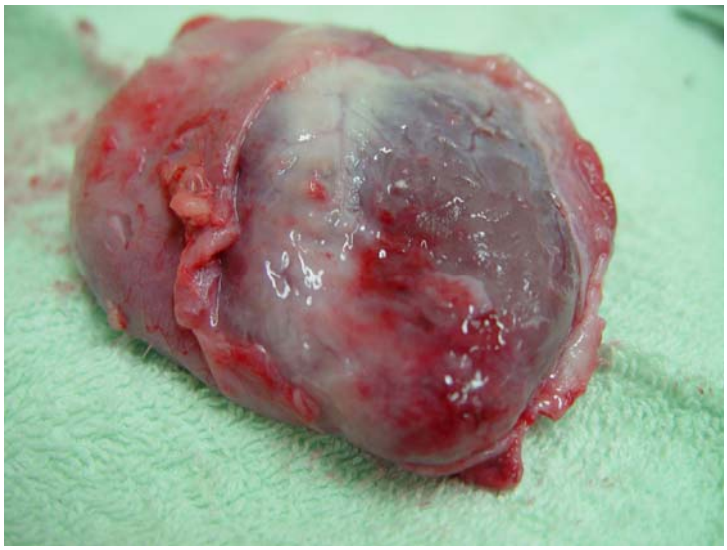

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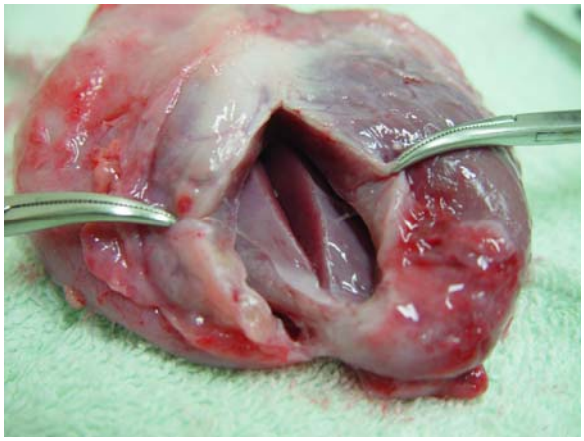


Fig. 2 Heart specimens. A. A heart specimen from a pig injected with normal saline. Absence of pericardial adhesion is evident. The needle is pointed to the site of coil in the anterior descending coronary artery. B. A heart from a pig injected with 4 mg/kg minocyclin. Pericardial adhesion is remarkable. C. Infarct area in cut-open view. The infarct is whitish and thin as comparing to the adjacent normal left ventricle wall.

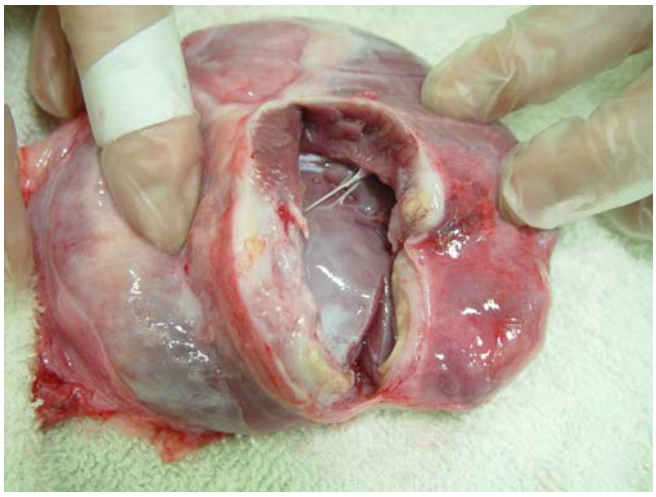
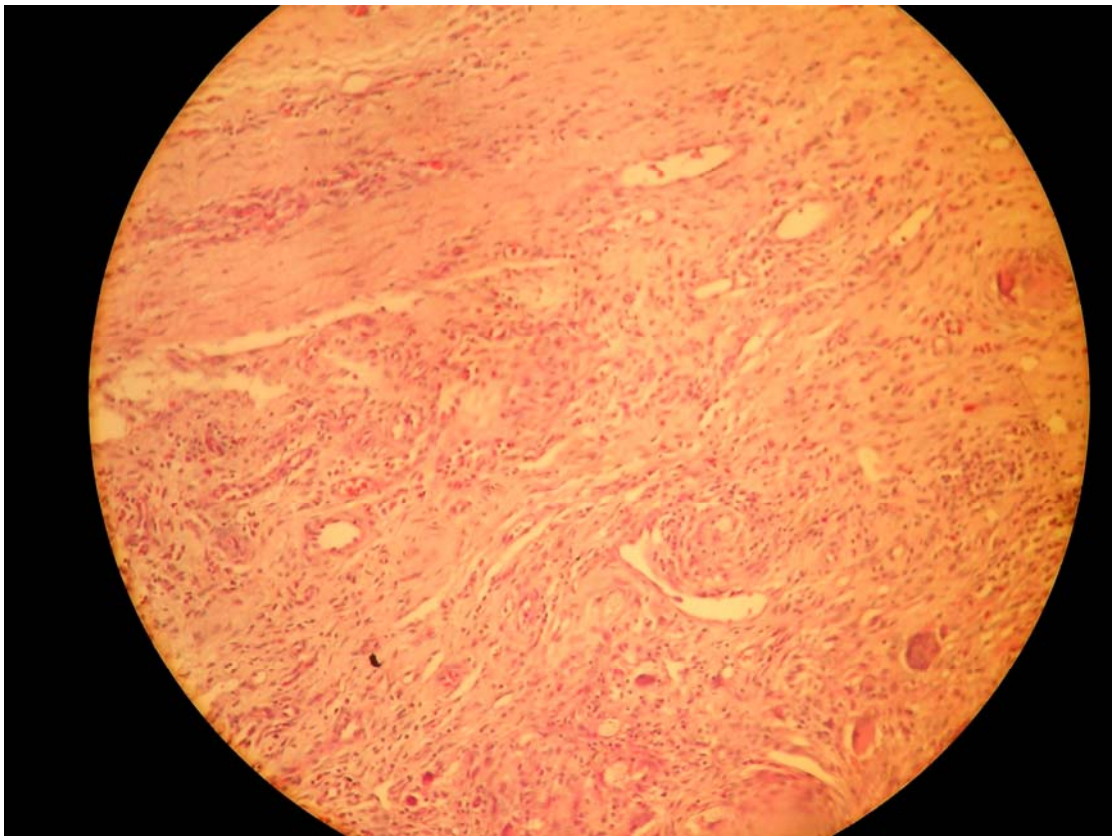


Fig. 3 An unusual change of the infarct area. The infarct becomes much thickened with yellowish, fat-appearance infiltration.

A



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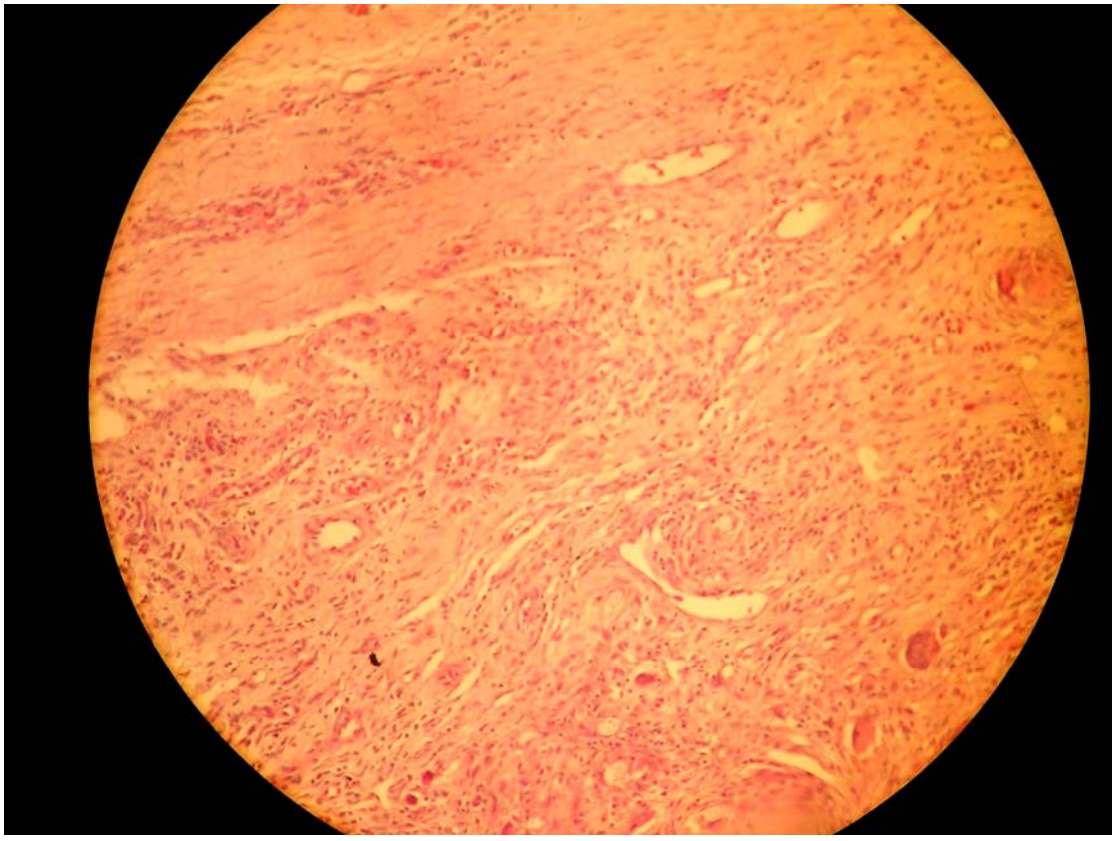

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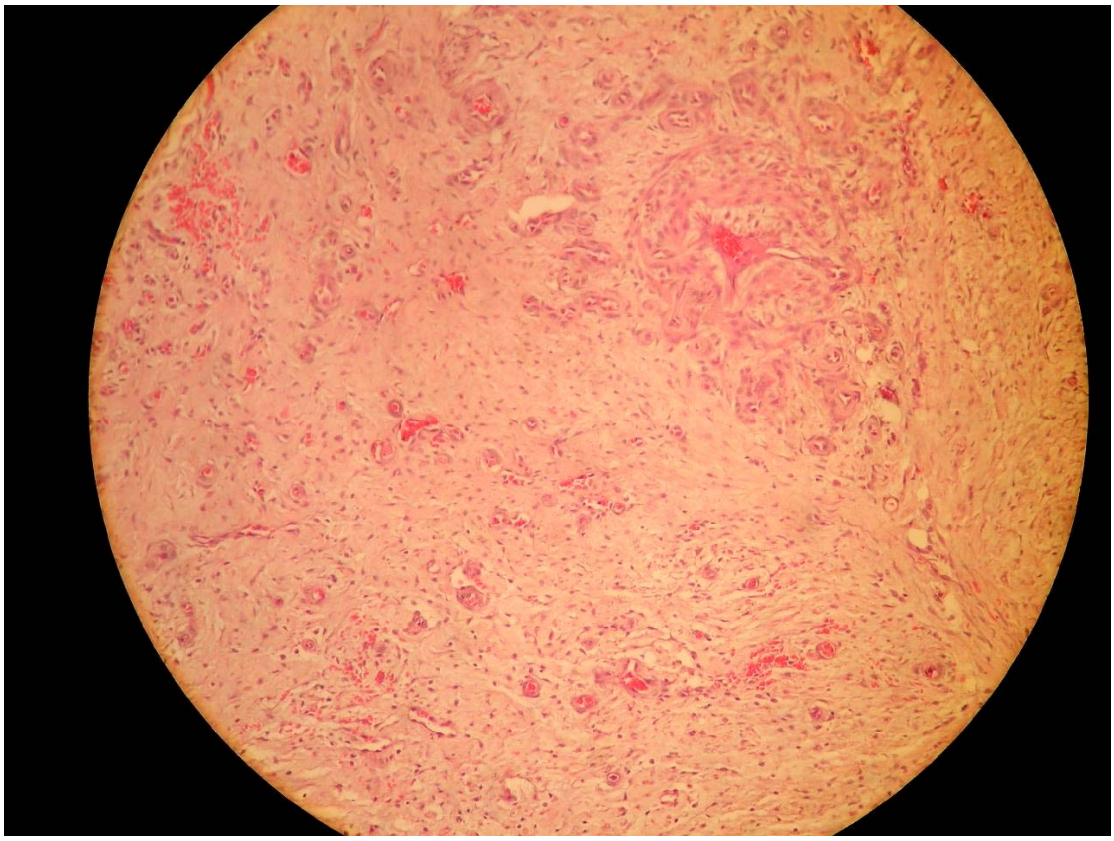

Fig. 4 Microscopic pictures of myocardial specimens. A, Infarct area from a pig of N/S group; B, a specimen of a pig from minocyclin 2 mg/kg group; C, a specimen of a pig from 4 mg/kg group. Magnification 200X.

Table 1 Pig number of the experiment groups

Group	Pig number
Normal saline (N/S)	5
Minocyclin 8 mg/kg	5
Minocyclin 4 mg/kg	7
Minocyclin 8 mg/kg	7

Table 2 Body weight, heart weight, pericardial adhesion and infarct size

Pig No.	Drug	Body Wt (kg)	Pericardial adhesion	Heart Wt (g)	Heart Wt corrected	Infarct size
1	N/S	9.8	No	65	6.63	moderate
2	NS	16.2	No	70	4.32	small
3	NS	11.4	No	80	7.02	moderate
4#	NS	9.6	No	90	9.38	moderat
5	NS	23.3	No	105	4.51	moderat
6*	M-8	19.4	minimal	120	6.19	moderate
7	M-8	13.4	mild	135	10.07	moderate
8	M-8	12.4	diffuse	100	8.06	small
9	M-8	18.1	diffuse	120	6.63	small
10	M-8	20.8	diffuse	130	6.25	small
11	M-4	16.7	diffuse			small
12	M-4	17.2	diffuse			moderate
13	M-4	13.5	diffuse	100	7.41	moderate
14	M-4	22.2	diffuse	145	6.53	small
15	M-4	15.5	diffuse	130	8.39	large
16	M-4	15.3	diffuse	100	6.54	small
17	M-4	12.3	diffuse	105	8.54	small
18	M-2	18	diffuse	120	6.67	small
19	M-2	15.6	diffuse	115	7.35	small
20	M-2	13.2	diffuse	100	7.58	moderate
21	M-2	14.4	diffuse	100	6.94	moderate
22	M-2	15.3	diffuse	135	8.82	small
23	M-2	14.1	diffuse	120	8.51	small
24	M-2	28	diffuse	150	5.36	small

N/S = normal saline; M denotes minocyclin; M-8, M-4, M-2 denote minocyclin 8 mg/kg, 4 mg/kg, 2 mg/kg, respectively; Heart Wt corrected = heart weight/body weight, g/kg

#Thickened infarct area with yellowish infiltrate

*Moderate pericardial effusion

Table 3 Mean body weight and heart weight by experiment groups

Group	Body Wt (kg)	Heart Wt (g)	Heart Wt corrected
N/S	14.06 ± 5.81	82 ± 16.05	6.37 ± 2.07
M-8	16.82 ± 3.72	121 ± 13.42*	7.44 ± 1.65
M-4	16.10 ± 3.19	116 ± 20.4**	7.48 ± 0.97@
M-2	16.94 ± 5.11	120 ± 18.03#	7.32 ± 1.17

*In comparing with control, $p<0.001$; **$p<0.005$; #$p<0.001$
@In comparing with control, $p<0.05$

計畫成果自評

本計畫在執行上相當成功，實驗動物共使用了 32 隻，其中有 24 隻完成實驗，成功率 75%。在引發急性心肌梗塞的實驗中，75%的動物能存活，並完成四星期之實驗期，算是很不錯的成績。

所有實驗動物均成功引發急性心肌梗塞，並依時間犧牲，取出心臟進行各種檢查。惟一遺憾是，在作組織免疫學染色時，無法找到對抗豬之特異性抗体，因而無法對血管作特殊染色。雖經一再努力，仍未能成功，且因而延誤了報告的繳交期。最後，只好以 H-E 染色的病理切片計算新生血管，準確度也打了折扣。

期望能儘快找到合適之特異性抗体，趕快完成血管計算，相信會有有意義的發現，而能將結果發表於好的醫學雜誌。又，所有豬的心臟檢体均仍保存於液態氮中，隨時可取出作進一步之檢驗。